Exhibit C

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Inducible gene expression in mammalian cells and transgenic mice

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Advances in biomedicine have accentuated the need to develop methods to deliberately modulate gene activity. In addition to improved versions of the system based on components of the tetracycline resistance operon, several strategies have recently emerged to control gene function at the transcriptional level. Particularly promising are approaches based on non-mammalian steroid hormones, and on small molecules that bind immunophilins.

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Abbreviations

CID	chemical inducer of dimerization
CsA	cyclosporin A
EcR	ecdysone receptor
FKCsA	FK506–CsA heterodimerizer
FRAP	FKBP12-rapamycin-associated protein
murA	muristerone A
NF	nuclear factor
rtTA	reverse tTA
RXR	retinoid X receptor
tetO	tetR binding site
tetR	tetracycline repressor protein
tTA	tetracycline transactivator
USP	ultraspiracle protein
VgEcR	VP16–EcR–glucocorticoid receptor hybrid

Introduction

The ability to manage the expression of genes introduced into mammalian cells and animals would further progress in many areas of biology and medicine. For instance, methods that allow the intentional manipulation of gene expression would facilitate the analysis of genes whose production cannot be tolerated constitutively or at certain stages of development. They would also be valuable for clinical applications such as gene therapy protocols, where the expression of a therapeutic gene must be regulated in accordance with the needs of the patient.

To be of broad benefit, gene regulation techniques must allow for rapid, robust, precise, and reversible induction of gene activity. An ideal system would fulfill the following requirements:

1. Specificity — the system must be indifferent to endogenous factors and be activated only by exogenous nontoxic drugs.

2. Non-interference — the components of the system should not meddle with cellular pathways.

3. Inducibility — in the inactive state, the basal activity of the system should be minimal, while in the active state it should quickly generate high levels of gene expression.

4. Bioavailability of the inducer — the regulating molecule should rapidly penetrate all tissues, crossing the placenta and the blood-brain barrier.

5. Reversibility — the inducer should be cleared swiftly from all tissues to allow the system to rapidly return to the inactive state.

6. Dose-dependence — the response of the system should be proportional to the concentration of the inducer, so that quantitative as well as qualitative problems can be addressed.

Early designs to direct gene expression in mammals were based on endogenous elements, such as cytokine response elements or heat-shock proteins. Due to a high level of basal expression in the uninduced state, and pleiotropic effects brought about by general inducing agents, these systems lacked the specificity required to regulate genes in mammalian cells and organisms. More advanced schemes have sought to avoid these problems by constructing switching mechanisms that rely on non-mammalian elements, or on reengineered mammalian proteins that are incapable of responding to endogenous inducers. The fundamental principle of these systems is the existence of a small molecule (the inducer) that modifies the activity of a synthetic transcription factor which, through a heterologous promoter, regulates the expression of a target gene. Increased specificity is achieved by selecting inducers that do not affect mammalian physiology, and by assembling chimeric transactivators with minimal homology to natural transcription factors and that do not interact with endogenous mammalian promoters. This review describes the basic features of these newer systems, with the understanding that a comparison of these strategies is hindered by the fact that they are often tested in different cellular contexts, and by the use of reporter genes/proteins of varying half-lives, which makes a comparative analysis of their kinetic properties difficult.

Tetracycline-based strategies

Building on studies in plants [1,2], which demonstrated that elements of the tetracycline resistance operon of the bacterial transposon *Tn-10* could regulate the expression

Figure 1
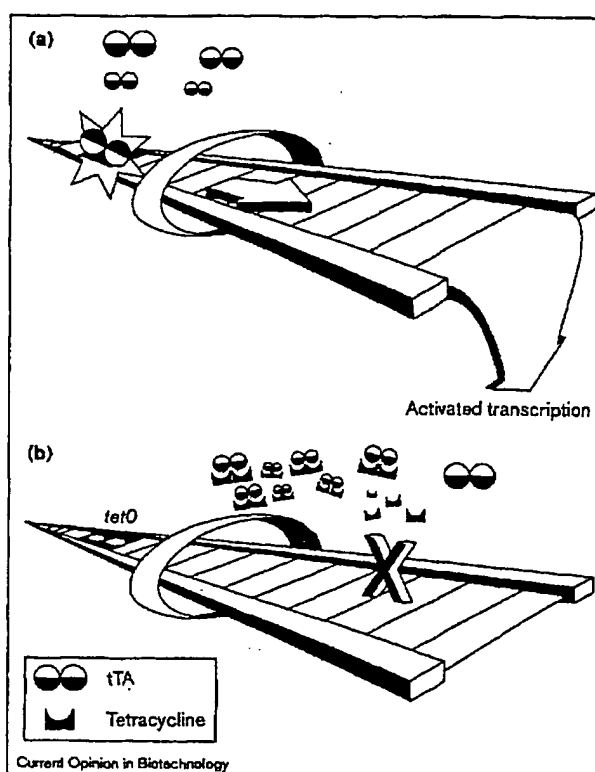
Tetracycline-repressible gene expression using tTA. (a) In the absence of tetracycline, a chimera of the tetracycline repressor and the VP16 transactivation domain (tTA), binds to tetO sites and activates genes expression. (b) Addition of tetracycline prevents tTA from binding, blocking expression of the target gene.

of eukaryotic genes, the tetracycline repressor protein (tetR) was adapted for use in mammalian cells [3]. This tetracycline-controlled system is based on the continuous expression of a fusion protein where tetR is converted into an activator by the addition of the transcriptional activation domain of the VP16 protein. In the absence of tetracycline, this chimeric tetracycline transactivator (tTA) activates gene expression through binding to a multimer of the natural tetR binding site (tetO) placed upstream of a minimal promoter. In the presence of tetracycline, the tTA undergoes a conformational change that prevents it from binding to the tetO sites, thereby arresting expression of the target gene (Figure 1). In the original description of this system [3], the expression of a reporter gene introduced into HeLa cells stably expressing tTA could be regulated over several orders of magnitude. Significant activation of gene expression was observed 25 hours after removal of tetracycline.

Because it presented significant advantages over existing approaches, the tTA system was quickly espoused as the preferred method of procuring inducible gene expression. Proteins of diverse function have been produced in a

tetracycline-dependent manner in cells stably expressing tTA [4–7]. As use of the tTA system spread, however, its limitations also became more apparent. A problem that was noticed during the development of the system was the toxicity of the tTA protein. The transactivator protein could not be detected in cells reliably expressing tTA, cells that efficiently responded to tetracycline [3]. The inability of cells to tolerate tTA expression has now been reported for a variety of cell types [8,9,10••,11,12]. While in cultured cells the toxicity associated with tTA expression presumably only encumbers the establishment of stable clones with proper tetracycline regulation, the deleterious effects of tTA expression seriously compromise the utility of this system for transgenic animals.

The tTA system under the control of the human cytomegalovirus promoter has been used to generate transgenic mice where the expression of reporter transgenes is modulated by tetracycline [13]. In the tissues that were tested, reversible expression of luciferase and β-galactosidase could be observed in the thigh muscle and tongue of these mice, but the level of transactivation or suppression upon tetracycline removal or administration was highly variable. This heterogeneous pattern of regulation can probably be ascribed to the stochastic behavior of tTA expression. On the one hand, the expression of tTA was found to be mosaic—not all cells/tissues carrying the tTA transgene could control the expression of reporter genes. On the other hand, the degree of regulation of indicator transgenes fluctuated greatly among animals, and remarkably, even between littermates derived from the same founder (i.e., mice with identical transgenes, integrated at the same chromosomal loci). Transgenic mice expressing the tTA from the mouse mammary tumor virus long terminal repeat displayed similar aberrations: the regulation of reporter transgenes was disturbingly heterogeneous, and the response of animals of the same line to tetracycline differed by as much as 53-fold [8]. This degree of variation was initially attributed to the fickleness of the viral promoters driving tTA expression, but experiments with tissue-specific promoters have encountered similar abnormalities [14].

For some in vivo applications, the instability of tTA expression will mean that to obtain transgenics suitable for experimentation, animals will need to be screened to select those expressing steady levels of tTA in a homogeneous manner. For other purposes, the irregularity of tTA expression may be quite acceptable [15,16]. For instance, a forebrain-specific promoter was recently coupled to the tTA system to control the expression of an activated form of calcium-calmodulin-dependent kinase II [17••]. Expression of this dominant mutant resulted in loss of hippocampal long-term potentiation, and spatial memory deficits. Tetracycline-mediated suppression of transgene expression reversed both phenotypes. This example suggests that the tTA system may be particularly well suited for gain-of-function studies where the transactivator

need only work in a subset of cells in order to generate a reversible phenotype.

To ameliorate the detrimental effects of tTA expression, tTA autoregulatory expression vectors have been created [9] where the tTA gene is placed under the control of a promoter containing *tetO* sites, such that in the absence of tetracycline tTA autoactivates its own expression (Figure 2). These strategies take advantage of the inherent leakiness of the tTA system to generate, in the absence of tetracycline, a positive feedforward loop that results in the maximum permissible levels of tTA expression. Because the amount of tTA is optimized, the absolute levels of expression of genes containing *tetO* sites are considerably higher than those obtained with the basic tTA system. In a functional assay, the autoregulatory tTA system induced tetracycline-dependent V(D)J immunoglobulin gene recombination at an order of magnitude higher efficiency than the original system, and in four times the number of clones. Transgenic mice were also generated by co-injection of the autoregulatory tTA cassette and a luciferase reporter, but the regulation of reporter transgenes was not improved in these animals.

Perhaps a more troublesome issue with the tTA system is the notable degree of basal expression that allows autoregulatory strategies to work. Basal expression can be the result of activation of the reporter constructs in the absence of bound transactivator, and/or of the inability of tetracycline to completely quell tTA transactivation. A strong level of basal expression limits the inducibility of the system, and forbids experiments with highly toxic proteins. Numerous investigators have described high levels of basal expression for the tTA system in cells and animals [8,11,13,18**,19–23]. In the case of stable clones and transgenic animals, some of this unintentional leakiness can be attributed to interference from the chromosomal regions into which the foreign DNA integrates. While all inducible systems are equally susceptible to integration effects, it is possible that the basal activity of the tTA system is due to the fact that this design requires the constant presence of tetracycline to efficiently suppress transcription, something that may not always be attainable, particularly *in vivo*.

This feature of the tTA scheme also limits its applicability, for there are situations where long term exposure to tetracycline may be undesirable or impractical. Furthermore, since in this system gene activation follows the removal of tetracycline, induction of gene expression is entirely dependent on elimination of the antibiotic. Because tetracycline deposits in bone, it has a slow rate of disappearance *in vivo* [24]. This kinetic constraint precludes the use of tTA when rapid inductions or pulsatile control are required. To increase the utility of the system, a mutant tTA protein has been isolated that displays the reverse properties of the original transactivator: it binds *tetO* sequences only in the presence of tetracycline (Figure 3) [25]. Within
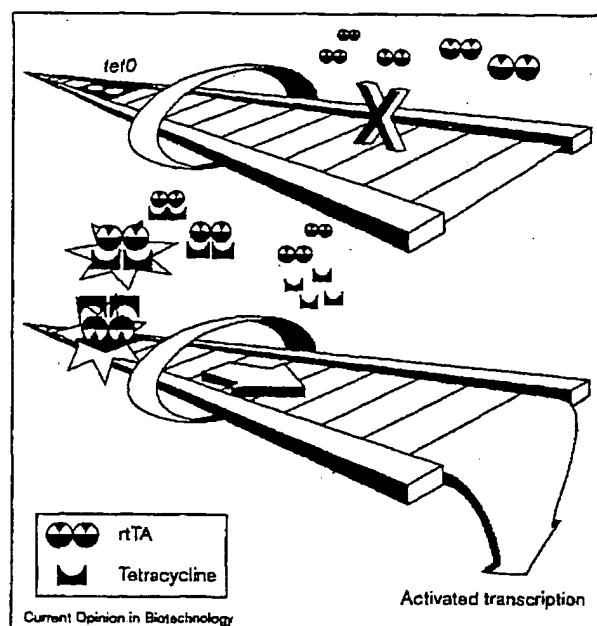
Figure 2
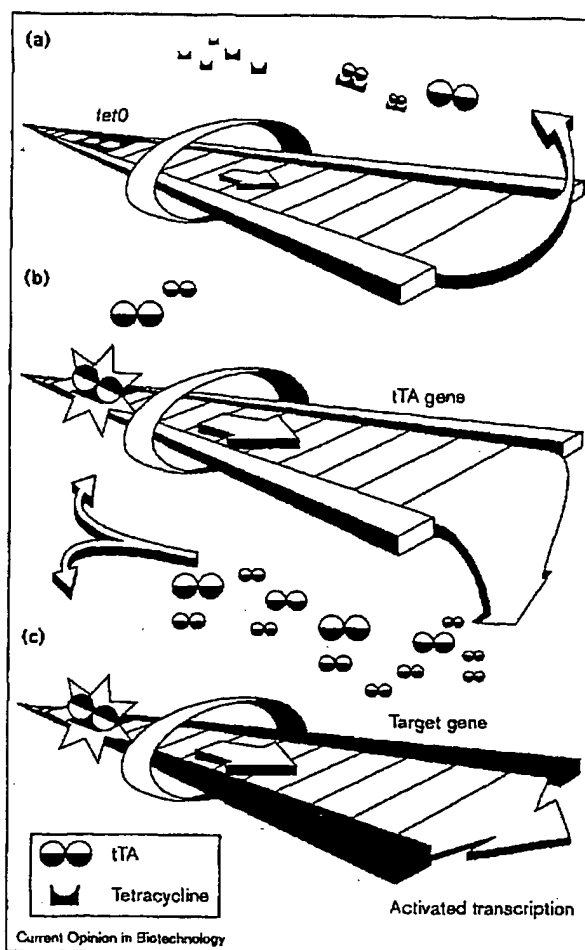
An autoregulatory tTA-inducible gene expression system. This is a modification of the tTA-based strategy. (a) The presence of tetracycline prevents tTA binding and activation of promoters containing *tetO*. Expression of tTA is under the control of *tetO* sites. (b) Upon removal of tetracycline, basal tTA expression generates a feedforward loop that induces autoactivation of tTA production, and (c) expression of the target gene.

24 hours after exposure to tetracycline or its derivatives (e.g., doxycycline), cells stably expressing this reverse tTA (rtTA) induced the expression of reporter proteins up to three orders of magnitude. Meaningful activation of target genes in transgenic mice expressing rtTA from the human cytomegalovirus promoter/enhancer required about nine hours of treatment with doxycycline; maximal expression was achieved after 24 hours [18**]. Interestingly, the response of specific organs to doxycycline treatment was quite distinct; differences were found in the time-course of transgene regulation and in the dose of doxycycline required to achieve inducible control. The kinetics of induction of reporter genes in tTA transgenics are also tissue-dependent: activation can be measured in some organs a day after doxycycline removal, while in

others reactivation of expression takes seven days or longer [17••,18••,26••].

Figure 3

Tetracycline-inducible gene expression using rtTA. rtTA is a mutated form of tTA that binds *tetO* sites in the presence of tetracycline, instead of in its absence. Hence, rtTA-based strategies are tetracycline-inducible rather than tetracycline-repressible. In the presence of tetracycline, rtTA binds *tetO* sites and activates *tetO*-containing promoters.

These observations substantiate concerns that had arisen regarding the efficacy of tetracycline schemes for all cell types and organs. There are cell lines where the tTA system does not work, and there are conflicting reports concerning the ability of tetracyclines to regulate gene expression in particular organs (e.g., muscle, kidney and brain) [8,11,13,14,18••,23,26••]. Cell-type-specific differences stress the need to calibrate the kinetics of tetracycline action for individual organs or tissues. The tetracyclines penetrate all tissues (including the brain and the fetal circulation), and they can be transmitted through the mother's milk [14,26••]. Doxycycline is reabsorbed in the renal tubules and the gastrointestinal tract, which confers on this isomer a longer half-life (12–24 hr) than that of tetracycline itself (4–12 hr) [27,28•]. *In vitro*, members of the tetracycline family are harmless in most situations: death of cultured cells is usually seen only at high doses of these antibiotics [25]. *In vivo*, these compounds deposit in the skeleton during gestation and throughout childhood, causing discoloration of the teeth and, in some cases, significant suppression of bone growth [29]. Doxycycline treatment during mouse development can also result in a

defect in neuronal maturation that spawns adult memory deficits [17••].

These pharmacokinetic properties indicate that inducible systems based on tetracyclines will be effective in a variety of settings, but they also exclude their use in situations where fast on/off interchange is required, or where their side effects may be unacceptable. A tetracycline antagonist (GR33076X) has recently been described [28•] that may prove useful for accelerating gene switching in tetracycline systems. Because this compound increases the DNA binding affinity of tTA, if it is administered at the same time that tetracycline is removed, the point where tTA starts binding DNA and inducing genes may be reached sooner. This kind of molecule may broaden the kinetics of tetracycline schemes in whole animals.

A variety of vectors have recently been created to facilitate the establishment of tetracycline gene regulation, and to expand the number of applications where it may be practical [19,26••,30,31]. One approach integrates transactivator and target gene units on a single plasmid, bypassing the uncertainties of separate integration effects and the tedious selection steps that accompany sequential transfection of tTA/rtTA and reporter plasmids [26••]. This single-plasmid design also simplifies the identification of transgenic mice strains with proper tetracycline-dependent regulation, as no crossing to reporter lines is required. When compared to bigenic mice created with the original tTA plasmids, the levels of induction of indicator genes in transgenics generated with this combined tTA vector were from 2–800-fold higher, perhaps because the single-plasmid approach ensures equal copy number of transactivator and target genes.

Viral vectors that ease the delivery of tetracycline systems and enable the analysis of whole cell populations have also appeared [20,21,32••,33•]. In one design, the target and transactivator components of the tTA system are arranged in opposition to each other on a single retroviral vector [32••]. In this configuration, high levels of tTA gene expression function not only to produce the transactivator, but also to decrease basal expression of the target gene by apparent antisense inhibition. The result is a population of infected cells where reporter genes are regulated with an impressive degree of inducibility. In another scenario, a bicistronic retrovirus that combines the one-vector approach with an autoregulatory tTA scheme has been used to generate and select populations of myoblasts that respond well to tetracycline induction [33•]. Retroviruses have also been the vector of choice for introducing tetracycline-regulated genes into cells that are later to be implanted into animals, an indirect test of the aptness of tetracycline-inducible systems for gene therapy. Efficient long-term regulation of erythropoietin secretion by the rtTA system in mice transplanted with retrovirally infected primary myoblasts has recently been demonstrated [10••], though a significant level of basal

erythropoietin secretion was noticed. In contrast, the tTA system was not useful in this instance, a reminder that the adequacy of any inducible system must be established for individual circumstances [22].

Hormone-modulated systems
Synthetic steroids – RU486

Two gene control systems based on components of mammalian steroid hormone receptors have been recently developed [34,35]. Steroid receptors are members of the nuclear receptor superfamily, ligand-dependent transcription factors that regulate gene expression by binding to short DNA sequences in the vicinity of target genes [36]. Nuclear receptors are modular proteins that consist of DNA-binding, ligand-binding, and transcriptional regulation domains. Created independently, these two steroid-based methods are nonetheless virtually identical: both combine a truncated form of the progesterone receptor hormone-binding domain with a yeast GAL4 DNA-binding moiety, and the transactivation domain of the VP16 protein. The mutated progesterone receptor moiety fails to bind progesterone, but it retains the ability to bind the progesterone and glucocorticoid antagonist mifepristone (RU486), such that in the presence of RU486 the fusion protein (called either GLVP [34] or TAXI [35]) activates transcription through a multimer of the GAL4 DNA-binding site placed upstream of a minimal promoter (Figure 4).

Although these systems represent an improvement over previous hormone-based designs, their performance in cells remains poor. In transient and stable transfections of various cell types, a high level of basal activity dampens the inducibility of these approaches, resulting in induction ratios that are rarely over 20-fold [22,34,35,37••]. The GLVP system appears to perform better in bigenic mice expressing GLVP in the liver and carrying a human growth hormone (hGH) target construct [37••]. Circulating levels of hGH increase significantly in these mice 8–12 hours after oral administration of RU486, but the response to RU486 diminishes over time. A similar blunting effect of repeated RU486 treatment has been observed in the TAXI scheme [35], raising concerns regarding the utility of these strategies for long-term protocols. Experiments with TAXI have also hinted at the possibility that these chimeric proteins may interfere with endogenous factors, an observation that could explain why it was difficult to generate GLVP-expressing transgenics [37••]. Moreover, even though the doses of RU486 required by these systems are below those known to antagonize progesterone, it may be prudent to search for safer analogs. In spite of these issues, an important advantage of steroid-based systems is that they appear to have more favorable kinetics than tetracycline approaches: these lipophilic hormones are quickly metabolized and have short half-lives *in vivo*. They may also penetrate less accessible tissues more efficiently.

Figure 4
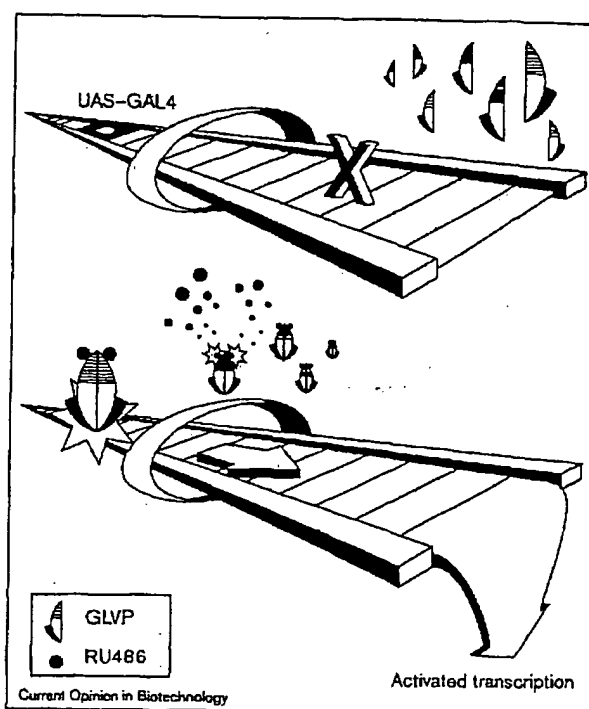
RU486-inducible gene expression. GLVP is a chimeric protein containing the VP16-transactivation domain, the GAL4 DNA-binding domain, and a mutated progesterone receptor ligand-binding domain. GLVP responds to progesterone receptor antagonists such as RU486 in lieu of progesterones. The inducible target consists of oligomerized GAL4 DNA-binding sites, a minimal promoter, and the gene of interest. In the presence of RU486, GLVP binds DNA in a hormone-dependent manner to activate gene transcription. UAS, upstream-activating sequence.

Non-mammalian hormones – ecdysone

A novel system based on the insect steroid ecdysone and its nuclear receptor exploits the auspicious kinetics of steroids, while eluding the potential complications of the use of a mammalian hormone as the inducer [38••]. During *Drosophila* molting and metamorphosis, a cascade of morphological changes is triggered by the steroid hormone ecdysone that leads to the degeneration of larval tissues and the appearance of adult structures. Mediating this response is the functional ecdysone receptor, a heterodimer of the ecdysone receptor (EcR) and the ultraspiracle protein (USP) [39]. Insect hormone responsiveness can be recreated in mammalian cells by cotransfection of EcR, USP, an ecdysone responsive reporter, and treatment with ecdysone or its analog muristerone A (murA), but the degree of induction under these conditions is rather unimpressive [40,41].

To increase the sensitivity of the system, a truncated ecdysone receptor was fused to the activation domain of VP16, and USP was replaced with its mammalian homologue, the retinoid X receptor (RXR) [38••]. In

cultured cells treated with murA, this receptor complex induced the expression of indicator genes up to four orders of magnitude. Upon murA administration, transgenic mice expressing the modified heterodimer in the thymus rapidly activated the expression of a reporter transgene. Though the EcR is not activated by mammalian hormones, to minimize the potential for interference with endogenous factors the specificity of the receptors and the response elements was further improved by creating EcR–glucocorticoid receptor hybrids, and a novel DNA-binding site for these complexes. In its final format, the VP16–EcR–glucocorticoid receptor hybrid (VgEcR) binds a composite synthetic response element (E/GRE) that is not recognized by natural nuclear hormone receptors (Figure 5). This highly artificial system retains the ability to efficiently induce the expression of reporter genes over four orders of magnitude in a dose-responsive manner. Stable expression of VgEcR and RXR has been attained in all cells and tissues attempted thus far, which suggests that overexpression of these proteins is not detrimental (D No, E Saez, RM Evans, unpublished data).

A salient attribute of this ecdysone-based method is the low level of basal activity that it exhibits. In a direct comparison, the VgEcR/RXR strategy demonstrated considerably lower basal activity than either the rTA or the rtTA systems, perhaps because in the absence of hormone, nuclear receptors that partner with RXR are known to exist in a complex with transcriptional corepressors [38••,39]. Combined with the use of a steroid inducer, this lack of basal activity allows for fast, robust inductions that can reach 1000-fold at the protein level within 6–8 hours after murA treatment of cells. In mice, half-maximal activation of a reporter transgene was observed about 16 hours after intraperitoneal injection of murA [42]. murA (which has nanomolar affinity for the ecdysone receptor) is bioavailable and well-tolerated by mice. This ecdysone analog is neither toxic nor teratogenic, and like ecdysone, which is completely excreted within 20 hours after administration, injected murA is rapidly distributed and eliminated [42,43]. These pharmacokinetic properties constitute a solid argument that the VgEcR/RXR system will continue to perform as a powerful and specific inducible system, specially for *in vivo* applications.

Gene regulation via induced dimerization of immunophilin domains

Another approach to regulate gene expression relies on a method of inducing protein dimerization that was derived from studies on the mechanism of action of immuno-suppressive agents [44•]. Compounds such as FK506 and cyclosporin A (CsA) subdue the immune response by binding with high affinity to the immunophilins FKBP12 and cyclophilin, respectively. These complexes interact with calcineurin to block T cell maturation [45]. Using a synthetic homodimer of FK506 (called FK1012), a general strategy was devised to bring together any two peptides, simply by endowing them with the domain

Figure 5
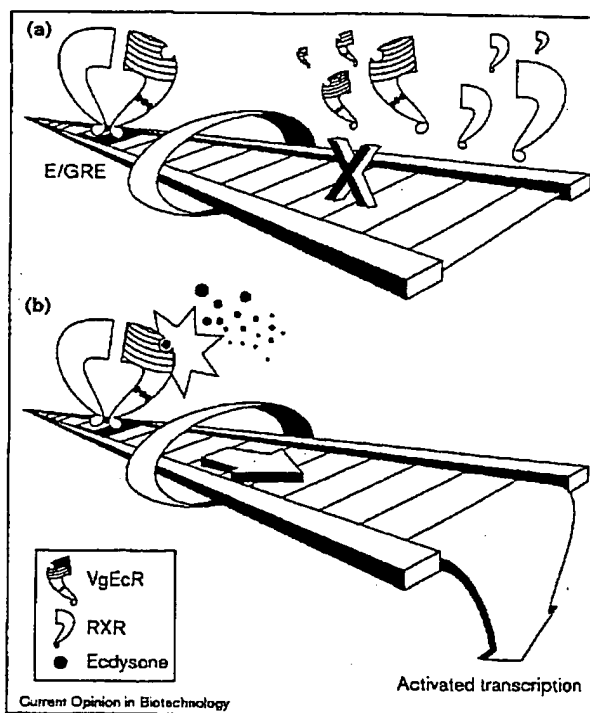
Ecdysone-regulated gene expression. (a) The functional inducible receptor is a heterodimer of VgEcR and RXR (retinoid X receptor). VgEcR is a truncated ecdysone receptor fused to the VP16 transactivation domain. The DNA binding specificity of the wild-type ecdysone receptor has been mutated so that it binds a novel response element. The target is comprised of modified ecdysone response elements (E/GRE), a minimal promoter, and the gene of interest. (b) In the presence of ecdysone (or the synthetic analog muristerone A), the VgEcR and RXR heterodimer activates transcription of the target gene.

of FKBP12 to which FK506 binds [46]. By chemically linking FK506 and CsA, a heterodimerizer molecule that can selectively connect two different immunophilin domains and their attached peptides was also generated [47••]. This FK506–CsA heterodimerizer (FKCsA) has been used to reconstitute a functional transcription factor by joining a GAL4 DNA-binding domain fused to FKBP12, and the transactivation moiety of VP16 bound to cyclophilin. In cells expressing these chimeric proteins, the expression of a promoter containing GAL4 binding sites was strongly stimulated in the presence of this 'chemical inducer of dimerization' (CID) FKCsA. Because FKCsA can only unite two different proteins, the use of this heterodimerizer CID avoids the formation of non-productive homodimers (e.g., one between two transcriptional activation domains).

The immunosuppressive drug rapamycin is a natural heterodimerizer that complexes with FKBP12 and FKBP12-rapamycin-associated protein (FRAP). A new inducible system based on rapamycin builds on the

614   Expression systems

Figure 6
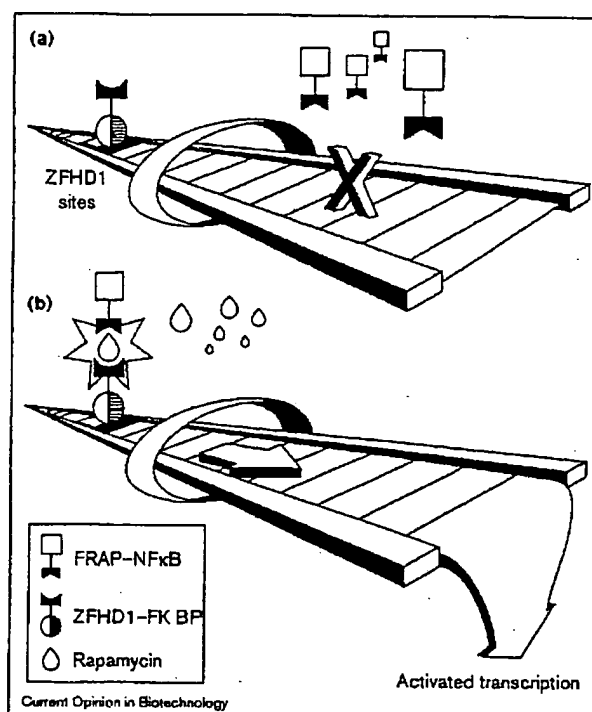
Rapamycin-mediated gene expression. (a) ZFHD1–FKBP is a chimera of FKBP and ZFHD1, an artificial DNA binding domain that binds to its own synthetic element. FRAP–NF-κB is a chimera of FRAP and the NF-κB transactivation domain. (b) In the presence of rapamycin, the FKBP and FRAP moieties dimerize, reconstituting a functional transactivator that induces transcription of a promoter containing ZFHD1-binding sites.

modularity of mammalian transcription factors and the heterodimerizing properties of this drug [48**,49]. In this design, ZFHD1 (an engineered transcription factor with a composite DNA-binding domain and novel DNA-recognition specificity [50]) was attached to FKBP12, and the activation domain from nuclear factor (NF)κB was bound to FRAP (Figure 6). Reassembled in cells upon the addition of rapamycin, this artificial transactivator induced the expression of a target gene three to four orders of magnitude in a dose-responsive manner, and with low or undetectable levels of basal expression. When implanted onto animals, cells stably transfected with this system efficiently regulated the expression of a reporter gene. Like other CIDs, rapamycin is a small molecule that enters many tissues (including the brain and the fetal circulation) and has a short half-life *in vivo*. Unfortunately, the attractive pharmacokinetics of this drug are compromised by its effects on the immune system: rapamycin cannot regulate gene expression at doses that are not immunosuppressive.

To address the problem of the interference of rapamycin and other CIDs with the functioning of either the immune system or of endogenous immunophilins, elegant structure-guided design has created more potent CIDs that no longer bind their natural targets [51,52**]. Novel receptor-ligand pairs have been developed by adding substituents to a CID that abolish binding to its endogenous partner, and then making the compensatory substitutions in the desired immunophilin to allow interaction once again. Several combinations of novel homodimerizing and heterodimerizing CIDs and mutated immunophilins with interacting surfaces that retain nanomolar affinity already exist [52**,53]. These modifications highlight the flexibility of CID-mediated approaches. Since these systems are completely modular, as long as they are based on the same CID, their components can be easily exchanged so that many different combinations can be examined. For example, multiple DNA-binding domains attached to the same immunophilin can be tested with the same transcriptional activation domain. An additional advantage of these approaches is that the monomeric form of a CID can sometimes compete with the dimerizer, accelerating the return of the system to the basal state [54*].

Conclusions

The enhancement of tetracyline-mediated techniques, and the development of methods based on innocuous steroids and CIDs, has increased the options of those seeking to attain inducible gene expression. That more problems have been identified in the tTA design than in others may simply reflect the fact that this system has been used in many more situations. As they are tested more extensively, newer systems are likely to display their weaknesses, for their perfomance has not been assessed in some demanding circumstances, such as in the context of a mixed population of infected cells. At the same time, these systems should profit from some of the same conceptual improvements that more established tetracycline schemes have gained from: the search for more potent inducers, the use of inducer antagonists to hasten gene switching, the development of streamlined vectors and novel delivery techniques, etc. Furthermore, the pharmacokinetic properties of these younger systems suggests that they may be useful for a wider range of applications than tetracycline-mediated schemes. To select a system, an investigator must focus on his/her particular needs, as the efficacy of individual methods is bound to vary dramatically in different applications. Someone interested in gene therapy may choose the one whose components display the least immunogenic potential, while someone interested in creating inducible knockouts may prefer the one that can deliver the most robust inductions in mice. Of course, simultaneous use of more than one of these systems will permit the regulation of several genes in the same cell, increasing the complexity of the questions that can be addressed experimentally.

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* •• of outstanding interest

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